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13. ABSTRACT Malaria continues as a major health threat throughout the tropical world and potential demand for antimalarials is higher than for any other medication yet the world faces a crisis--drug resistance is emerging and spreading faster than drugs are being developed and the flow in the pipeline of new drugs has all but stopped. This represents a particular threat to the US Military. A new strategy for drug development is urgently needed. Current drugs are based on a small number of target molecules or lead compounds and in most cases the target of drug action is yet to be identified. Resistance is emerging rapidly and the mechanisms of resistance are poorly understood. The identification of new targets or new candidate drugs, based on an understanding of the parasite biology, are key elements in this new strategy. The goal of this work is to use a molecular genetic approach both in the identification of new drug targets and in the investigation of mechanisms of drug resistance. There are two parallel approaches being developed, one the development and characterization of a homologous transformation system, and two the development of a heterologous expressions system in yeast for potential drug target enzymes. The yeast expression system should allow rapid screening of new drugs, greatly increasing the rate at which new antimalarials can be tested and developed. Both of these approaches are based on the functional analysis of malaria genes with goal of using this information in the identification and development of new antimalarial drugs. The recent initiation of the *Plasmodium falciparum* genome project will complement this project in that new potential targets will be identified and the methodologies developed because of the crisis facing us in antimalarial drugs.

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(5) Introduction

Malaria continues as a major health threat throughout the tropical world and potential demand for antimalarials is higher than for any other medication yet the world faces a crisis-drug resistance is emerging and spreading faster than drugs are being developed and the flow in the pipeline of new drugs has all but stopped. This represents a particular threat to the US Military. In a short time there may be parts of the world where no effective antimalarial drug is available. The recent emergence of multidrug resistant malaria parasites has intensified this problem. Recognizing this emerging crisis, it is necessary to identify new strategies for the identification and development of new antimalarials. The goal of this work is the development of a framework for antimalarial drug development into the 21st century.

A new strategy for drug development is urgently needed. Current drugs are based on a small number of target molecules or lead compounds and in most cases the target of drug action is yet to be identified. Resistance is emerging rapidly and the mechanisms of resistance are poorly understood. The identification of new targets or new candidate drugs based on an understanding of the parasite biology are key elements in this new strategy. Clearly the development of a new antimalarial will require both basic and applied research working in concert with one another.

The goal of this work is to use a molecular genetic approach both in the identification of new drug targets and in the investigation of mechanisms of drug resistance. There are two parallel approaches being developed, one the development and characterization of a homologous transformation system and two the development of a heterologous expressions system in yeast for potential drug target enzymes. The yeast expression system should allow rapid screening of new drugs, greatly increasing the rate at which new antimalarials can be tested and developed. Both of these approaches are based on the functional analysis of malaria genes with the goal of using this information in the identification and development of new antimalarial drugs. The development of these tools should facilitate future drug development and allow us to translate our molecular genetic knowledge into the practical identification and development of new antimalarials. This is a new strategy and it is being applied because of the crisis facing us in antimalarial drugs. The previous strategy, namely lead directed screening must be supplemented by new strategies or we will be faced with multiresistant *Plasmodium falciparum* and no drugs to treat it.

Malaria represents a major and increasing threat to the U.S. Military. Many of the sites of current or potential U.S. Military involvement are endemic for malaria and in several sites, multidrug resistant *P. falciparum* represents a major problem especially for non-immune military personnel. Current drugs available to the U.S. Military are quickly losing their effectiveness because of emerging and spreading drug resistance. This work is directed both at identifying new drugs and drug targets, but equally importantly toward an understanding of drug resistance mechanisms with the goal of preventing or overcoming drug resistance in the malaria parasite.

(6) Body

The goal of this work is to use a molecular genetic approach both in the identification of new drug targets and in the investigation of mechanisms of drug resistance. There are two parallel approaches being developed: one, the development and characterization of a homologous transformation system and; two, the development of a heterologous expressions system in yeast for potential drug target enzymes. The yeast expression system should allow rapid screening of new drugs, greatly increasing the rate at which new antimalarials can be tested and developed. Both of these approaches are based on the functional analysis of malaria genes with goal of using this information in the identification and development of new antimalarial drugs. The development of these tools should facilitate future drug development and allow us to translate our molecular genetic knowledge into the practical identification and development of new antimalarials. This is a new strategy and it is being applied because of the crisis facing us in antimalarial drugs. The previous strategy, namely lead directed screening must be supplemented by new strategies or we will be faced with multiresistant Plasmodium falciparum and no drugs to treat it.

These goals will be accomplished through the following technical objectives:

A. Functional analysis of putative drug resistance genes and new drug target genes in the malaria parasite through the further development of a transformation system for the malaria parasite including:

1. Development of methods to express and modify parasite genes

Development of methods for stable cloning of *Plasmodium falciparum* sequences in *E. coli*

A major technical obstacle in all malaria cloning projects is the instability of many *P. falciparum* sequences in standard *E. coli* based host-vector systems. Thus, the goal of this work is to identify a convenient bacterial cloning system in which *P. falciparum* DNA sequences are stably incorporated and maintained. This will complement ongoing work by others to use yeast cloning systems, including YACs, the development of bacterial artificial chromosomes (BACs) and the development of shotgun libraries using small fragments of DNA.

The underlying hypothesis in this work is that there is a specific mechanism(s) in *E. coli* for recognizing and deleting "unstable" *P. falciparum* sequences. The *P. falciparum* DNA is stable in plasmodium itself and appears based on the data derived from YAC clones to be relatively stable in yeast. Thus, the DNA is not inherently unstable in all systems, but in some way is recognized in *E. coli*. The goal of these experiments is to discover that mechanism and then attempt either to modify the *P. falciparum* DNA or generate *E. coli* strains in which *P. falciparum* DNA can be readily cloned.

We first tested the hypothesis that the instability of *P. falciparum* DNA cloned into plasmid vectors is due to its interaction with the enzymes of one of the major recombination pathways in *E. coli*. Extensive research has been done in this area and *E. coli* strains with mutations in each of the key enzymes are readily available. Thus, the first set of experiments will use these mutant strains in cloning experiments designed to test for increased stability of *P. falciparum* DNA. Among the major recombination pathways active in wild type *E. coli*, the Rec BCD pathway and the Rec F pathway are specific for conjugational and plasmid recombination pathways, respectively. Both of these pathways involve the Rec A enzyme, which is central to the initiation of recombination. Extensive work on mutations in enzymes central to these two major pathways has demonstrated that there is significant redundancy and overlapping functions in the recombination pathways. Thus, the absence of one enzyme can shift both the rates of chromosomal and plasmid recombination and also result in a different constellation of enzymes used for each type of recombination. Since, in the case of the *P. falciparum* DNA instability problem, we do not know which, if any, of these pathways or enzymes are involved, we screened *E. coli* strains with mutations in each of the major enzymes and in some cases, with mutations in more than one enzyme in the pathway.

We first concentrated on mutations in the plasmid recombination pathway, the RecF pathway. We have received from Dr. Richard Kolodner three *E. coli* strains with mutations in this pathway, one in the rec F gene and two in the rec J gene. Each of these strains is defective in plasmid recombination. We have designed a test plasmid system in which we have ligated a segment of *P. falciparum* DNA known to undergo extensive rearrangement in our standard HB101 *E. coli* cells. The DNA fragment, the pfmdr1 gene, to be used in these studies has been extensively characterized in my laboratory. We have diagnostic restriction digests to detect rearrangement in any region of the DNA and have PCR assays for finer sequence analysis

For each mutant strain, we tested the same ligation product; the pfmdr1 coding region fragment ligated to a pBluescript based backbone. We produced competent *E. coli* and transfected them using electroporation. Each strain was transformed with 10 ng of ligation product. As a control in each experiment, a ligation reaction using a fragment of Leishmania enriettii DNA of similar length and encoding a similar protein product was included. Transformants were scored, transferred to nylon filters for hybridization analysis and positive colonies were picked for miniprep/agarose gel analysis. For each *E. coli* strain, greater than 500 colonies were analyzed. Approximately 1% of the colonies, which were derived from the pfmdr1 ligation, contained insert DNA whereas greater than 85% of the colonies derived from the control ligation contained insert. This result confirms our previous non-systematic observation of the extreme lability of this fragment of *P. falciparum* DNA in *E. coli*. In addition, for those colonies that had inserts, none had full-length inserts for the falciparum DNA while all of the positive control ligation plasmids contained full-length insert. The results were regardless of *E. coli* strain tested, mutations in the Rec A, Rec BC, Rec F and Rec J loci made no difference in these results. Thus, based on this initial work, we concluded that single mutations in key enzymes in either the plasmid or chromosomal recombination pathways did not result in any changes in the outcome of the transformation experiments.

During the course of these experiments, we made an interesting observation. We recovered plasmids with different sized inserts of the pfmdr1 gene product, ranging from nearly full-length (3.8 kb) to small inserts of less than 1 kb. After the screening of more than 3000 colonies, we recovered a plasmid that contained the full-length insert DNA. (Note: this was independently confirmed based on sequence) Once these plasmids had been grown in *E. coli*, they could be stably transfected and propagated in any of the *E. coli* strains tested without any further rearrangement or deletion. Thus, these Plasmodium falciparum sequences were stable in *E. coli* once closed circular plasmids had been formed. This led us to the hypothesis that it was not the sequence of this region of DNA per se, but instead something that was different between the ligation product and supercoiled DNA. One obvious difference was the physical structure of the DNA and another was the topology. Plasmid purified from *E. coli* is supercoiled, with no nicking in the DNA strands. In contrast, the ligation reaction is a mixture of DNA circles and linear molecules. Even in a ligation reaction that has gone to completion, there remain two nicks on the DNA, one at each cloning site where the vector DNA has been dephosphorylated to reduce the background. There is no supercoiled DNA in these ligation reactions.

To test the role of topology and to potential role of nicked DNA in this instability reaction, we performed the following experiments. We purified supercoiled plasmid DNA containing the full-length pfmdr1 insert fragment. We then treated the plasmid with DNase I under conditions of partial digestion. This DNA was purified and used to transfect *E. coli* rec A cells. Transfection with untreated or mock-treated DNA resulted in the recovery of the input plasmid DNA with no deletion or rearrangement, while transfection with those plasmids which had been treated with DNaseI prior to transformation, resulted in plasmids which had substantial deletions in the pfmdr1 sequence and in some cases, deletions in the plasmid DNA. Thus, we conclude from these experiments, that it is not the sequence of the *P. falciparum* DNA per se, but instead a combination of the sequence and its physical state. One interesting finding is that as the DNase I concentration was increased, the number of transfected colonies recovered decreased. This decrease was greater than predicted based on the recovery of DNA and may indicate that multiply nicked DNA has a greatly decreased efficiency of transformation. This may in some part explain why the overall cloning efficiency of *P. falciparum* DNA is lower than that of other DNA. Because of its high AT-content, the DNA is more susceptible to nicks and thus may be a substrate of rearrangement and may have a reduced efficiency of cloning.

However, based on our analysis of bacteria defective in recombination enzymes, we concluded that if the *P. falciparum* DNA was the target of the recombination pathway in *E. coli*, it must be recognized by more than one enzyme or by an enzyme not defective in any of these strains. We then tested *E. coli* strains with multiple defects in recombination pathways and DNA repair mechanisms. Of particular interest is the SRB strain, originally obtained from Strategene, and the commercially available SURE cells. Ligation product cloned into these cells an interesting set of results, in the SRB strain, greater than 95%of the plasmids isolated from colonies which contained insert had a full

length insert, whereas in those plasmids isolated from SURE cells there was evidence for partial deletion or rearrangement of the Plasmodium sequence. This result has now been repeated with different Plasmodium falciparum DNA fragments and the plasmids isolated from SRB transfectants consistently contain full-length insert. In comparing the genotype of SRB and SURE, the only published difference is in the recB locus. The SURE cells are defective in the recB pathway while in SRB cells; the recB enzyme is intact. However, during our initial experiments, we directly compared recB defective and intact cells and saw no difference in the cloning of full-length pfmdr1. Therefore, other components in the SRB/SURE genotype must be playing a role in the stabilization of these *P. falciparum* sequences.

Cloning of *P. falciparum* genes in expression vectors

Using the methods described above, we have now successfully cloned the Plasmodium falciparum PfMDR1 gene and as a fusion gene with Green Flourescent Protein in the pHC1 plasmid developed by Alan Cowman and coworkers. This plasmid contains a selectable marker (the T. gondii DHFR gene resistant to pyrimethamine) and an expression site. Transfection experiments using this plasmid construct are now in progress.

2. Development of methods for targeted gene disruption

These experiments are still in the planning stages, pending the outcome of the transfection experiments described above.

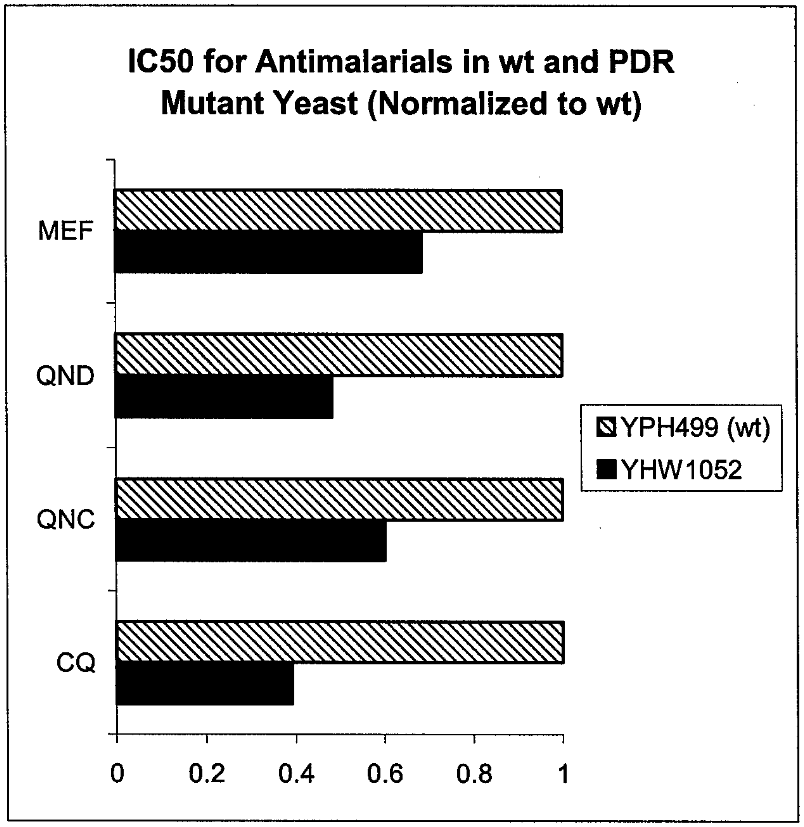
B. Functional analysis of putative drug resistance and new drug target genes in the heterologous yeast expression system

1. Identification of new drug target genes through complementation analysis in yeast. A single yeast strain with mutations in PDR5/10/SNQ2 has been chosen for this work.

ABC transporters mediate drug resistance in yeast

PDR5, PDR10 and SNQ2 confer resistance to many xenobiotic compounds and yeast strains deleted in these genes are hypersensitive to many different substances. The strategy in this series of experiments is similar to the complementation of the ste6 defect, namely, the complementation of a yeast strain deleted for the PDR5, PDR10 and SNQ2 genes. We have discovered that this yeast strain (YPH1052) is sensitive to several antimalarial including, mefloquine (MEF), quinidine (QND) quinicrine (QNC) and chloroquine (CQ) in contrast to the parental strain (YPR 499) which is resistant to these drugs as shown in below.

Figure 1



In initial proof of principle experiments, a pYESLe expression library was used to complement the YRP1052 strain under chloroquine selection and 30 independent colonies were recovered. Analysis of these colonies demonstrated that they all contained the same gene. Analysis of that gene is now in progress. Construction of a pYESPf library has now been initiated, using the recently adapted method of cDNA synthesis we have developed for SAGE analysis. In the initial experiments, selective drugs will include cyclohexamide. This drug is highly selective for yeast expressing complementing ABC-transporters and will give the lowest background. This approach has been used to identify genes from Candida albicans, which complement the PDR5/SNQ2 defect, and these are ABC-transporters (Sanglard, Kuchler et al. 1995). Parallel experiments using chloroquine as the selective drug will also be initiated.

As above, the limitations of these experiments are that the parasite gene must function in yeast to confer resistance. This experiment is dependent on resistance as the positive selection marker. We have already demonstrated that both the PfMDR1 gene and the LeMDR1 gene can modulate drug sensitivity when transfected in yeast and thus anticipate that other parasite genes will also function in yeast. The major advantage of this functional selection system is that we will know the functionality of the transporter before we begin characterization. In addition, this system allows us to screen for genes that confer resistance to many different types of drug including known antimalarial drugs.

Analysis of the transcriptional response to drug treatment

In parallel with this work, we have undertaken a collaboration with the WRAIR group to analyze the response of yeast strains YPR 499 and YPH 1052 to antimalarial drugs using the method of whole genome expression analysis. The goal of this project is to determine the inherent response of cells to treatment with antimalarial drugs. The project addresses several interrelated questions: first, what is the global response of the a cell when it is treated with a cytotoxic drug? Is there a set of genes whose expression is changed upon drug treatment and are they unique for each drug or are there common genes expressed at higher or lower levels during cell death? These fundamental questions relate directly to the development of drug resistance and have not been addressed at the molecular level in *Plasmodium falciparum*.

The recent availability of complete genome sequences and methodologies to scan whole genomes has allowed investigators to ask questions about the global response of cells to various stimuli (Heller et al., 1997; Schena et al., 1995; Schena et al., 1996; Velculescu et al., 1995). Much of the initial work was done in *Saccharomyces cerevisiae* and has led to the surprising observation that 30 to 40 genes change in expression levels when cells encounter toxic drugs or nutrient levels change (DeRisi et al., 1997). These results imply that response to drug treatment involves the interaction of several gene products and several pathways may exist by which the cell can resist the toxic effects of the drug. Over time, a particular pathway may predominate in resistant cells, but the expression of other proteins may continue to play an important role. In yeast, more than 20 genes are turned on after treatment with phorbol ester implying a specific transcriptional activation and thus opening a new avenue for the development of interventions.

We will use as a the Affymetrix Yeast GeneChip system as a model system for the first phase of this work. We have chosen to use the yeast system for several reasons. First, it is a well-characterized system and the tools for whole genome scanning are now available in a readily usable format. In addition, genes associated with drug response and resistance have been identified and we have yeast strains that have one or more of these genes disrupted. One of the interesting observations which has appeared in the first analysis of gene expression in yeast after xenobiotic treatment is the rapid upregulation of the PDR5 gene, a member of the ABC transporter gene family. We have courtesy of Dr. Karl Kuchler, yeast strains that have "knockouts" in one or more of the ABC-transporters and we will examine these for their response to various drugs. The goal of the first segment of this project is to investigate the global response of Saccharomyces cerevisiae to treatment with antimalarial drugs as a model system for detection of drug response expression profiles in *P. falciparum*.

The first set of experiments will determine the whole genome expression pattern in S. cerevisiae treated with the antimalarial drug chloroquine. We have previously discovered that a yeast strain YPH1052 which has disruptions in three ABC-transporter genes (PDR5, PDR10 and SNQ2) is highly sensitive to chloroquine when compared to (YPR499) parent strain and have proposed a model in which the lack of chloroquine susceptibility in the parent strain is mediated by expression of these three ABC-

transporters. In order to test this model, we have begun whole genome expression analysis of the two yeast strains. We will now begin experiments to examine gene expression of each strain in the presence of chloroquine (10 mg/ml).

To best decipher the patterns of gene expression in such a complex setting, it is imperative to monitor the expression level of genes simultaneously. The Walter Reed Army Institute of Research (WRAIR) Gene Array laboratory has explored the use of high density DNA arrays to monitor the expression of genes associated with the pdr and ABC transporter cascades in yeast constructs. To date, the technique has not only confirmed the established markers of multiple drug resistance in both sensitive and resistant strains but has also identified several expression patterns not previously identified. In addition, this effort has reported the complete inventory the transcriptome of yeast strains YPH 499 and YWH 1052 for the first time.

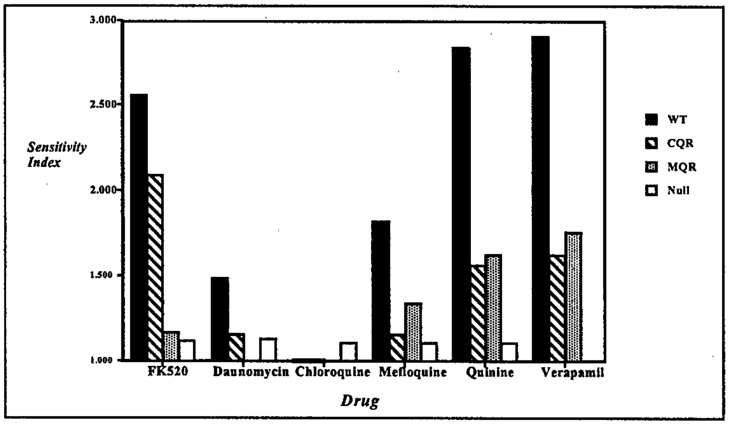
2. Development of new, rapid, high throughput drug screening methods for malaria genes expressed in yeast

Yeast as an expression system for measuring ABC transporter function

A heterologous yeast expression system was developed to test the function of ABC transporters such as PfMDR1 in a mechanism of drug resistance and to develop a tool for rapid testing of agents that circumvent drug resistance in malaria. Both protein localization and protein structure are proposed to be important for mediation of drug resistance by PfMDR1, and changes in these parameters are predicted to alter either the level of drug resistance or the pattern of substrate specificity. To examine how PfMDR1 functions as a transport molecule, we expressed the gene in a heterologous yeast system, and took advantage of homology between PfMDR1 and other ABC transporters. Two phenotypes can be examined by this yeast assay system, including mating and drug responsiveness. Yeast have approximately 30 ABC transporters, one of which, STE6, mediates export of mating pheromone **a**-factor from a-haploid cells. Without *ste6* expression yeast cannot export **a**-factor and cannot mate. Transformation of yeast deficient for *ste6* with an expression plasmid containing PfMDR*1* sequences demonstrated that expression of PfMDR*1* in yeast deficient for *ste6* restores this mating phenotype (Volkman et al., 1995). Thus, complementation of *ste6* demonstrates that PfMDR1 functions as a transport molecule in this yeast system and mediates the export of **a**-factor to restore mating.

We next wanted to understand if this yeast assay system could be used to address the function of PfMDR1 in the modulation of drug resistance (Volkman et al., 1999). Yeast transformed with either wild-type or mutant forms of PfMDR*1* were assayed for growth in the presence of several different of drugs, including antimalarial drugs and resistance reversers. These data argue that PfMDR1 can modulate the response of yeast to drugs and that mutations associated with drug resistant *P. falciparum* alter this level of response. Furthermore, that these mutations may be important for determining the specificity of substrates that can be transported by PfMDR1.

Figure 2: Yeast Drug Assay



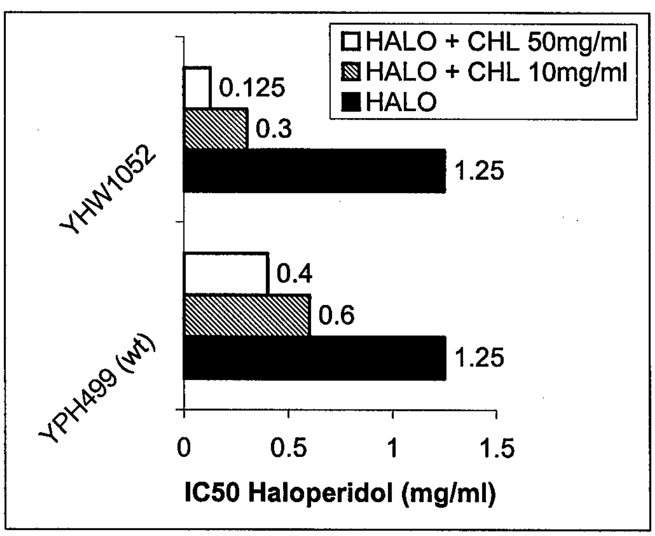
Transformants were grown in the absence or presence of drug at various dilutions over an 18 to 20 hour period. Growth was measured by OD and expressed as a response index, calculated as the ratio of growth in the absence of drug to the growth in the presence of drug, normalized to the vector only transformant. For all compounds, the vector only transformant grew equally well in the presence or absence of drug.

These data argue that PfMDR1 functions as a transport molecule and that this yeast assay system can be used to test the functional requirements of PfMDR1. Finally, this assay system can be used to screen for compounds that interact with PfMDR1 and for the identification of combinations of therapeutic agents that modify these drug responses.

Development of a resistance reversal assay in the yeast system

Based on the above results, we have initiated experiments to develop a high-throughput screening in the yeast system for resistance modulation. The system has now been adapted for 96-well plates and results from a pilot experiments is presented below. Both YPR 499 and YPH 1052 are more sensitive to a combination of chloroquine and haloperidol than to either drug alone. Thus, drug sensitivity in yeast appears to be modulated by a known resistance reverser. These strains are now being tested with several additional known resistance reversers.

Figure 3
Reversal of Drug Resistance with Haloperidol



(7) Key Research Accomplishments

- Development of an efficient system to clone *P. falciparum* sequences in *E. coli* for development of transfection and expression vectors

- Complementation analysis of yeast strains deleted in PDR5/10/SNQ2 with parasite genes

- Development of a high throughput assay for testing resistance modulators

(8) Reportable Outcomes

Manuscripts

Volkman SK, Emerson LR, Woodcock SA, and Wirth DF. 1999. Expression of the *PfMDR1* gene of *Plasmodium falciparum* Modulates Drug Resistance in Yeast. In preparation.

Mbacham WF, Daily J, Golightly L, Budge E, harris D, Ruel K and Wirth DF. 1999. Identification of putative promoter elements in *P. gallinaceum*. In preparation.

Abstracts

Volkman SK, Larry M.C. Chow LMC, Emerson LR and Wirth DF. 1998. Using a yeast complementation system to identify novel drug resistance genes and assay transport function in *Plasmodium falciparum*. American Society of Tropical Medicine and Hygiene, San Juan, Puerto Rico.

Patankar S, Mbacham WF and Wirth DF. 1998. PGS28: Regulation of gene expression and protein targeting in *Plasmodium gallinaceum* ookinetes. American Society of Tropical Medicine and Hygiene, San Juan, Puerto Rico.

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(9) Conclusions

Progress has been made in several areas of the project. The use of yeast as a model system for testing parasite genes has proven very valuable and will provide new insights into the mechanism of drug resistance and drug response. The transfection system for Plasmodium falciparum remains cumbersome but recent progress is encouraging.

The goal of this work is to use a molecular genetic approach both in the identification of new drug targets and in the investigation of mechanisms of drug resistance. Two parallel approaches are being developed based on the functional analysis of malaria genes with the goal of using this information in the identification and development of new antimalarial drugs. The development of these tools should facilitate future drug development and allow us to translate our molecular genetic knowledge into the practical identification and development of new antimalarials. This is a new strategy and it is being applied because of the crisis facing us in antimalarial drugs. The previous strategy, namely lead directed screening must be supplemented by new strategies or we will be faced with multiresistant *Plasmodium falciparum* and no drugs to treat it.

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